# Change of X Chromosome Status during Development and Reprogramming

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**ABSTRACT** : X chromosome inactivation (XCI) is a process that enables mammalian females to ensure the dosage compensation for X-linked genes. Investigating the mechanism of XCI might provide deeper understandings of chromosomal silencing, epigenetic regulation of gene expressions, and even the course of evolution. Studies on mammalian XCI conducted with mice have revealed many fundamental findings on XCI. However, difference of murine and human XCI necessitates the further investigation in human XCI. Recent success in reprogramming of differentiated cells into pluripotent stem cells showed the reversibility of XCI *in vitro*, X chromosome reactivation (XCR), which provides another tool to study the change in X chromosome status. This review summarizes the current knowledge of XCI during early embryonic development and describes recent achievements in studies of XCI in reprogramming process.

**Key words** : X chromosome inactivation, XIST, XCI, XCR, Reprogramming

X chromosome inactivation (XCI) is a process by which mammals compensate gene dosage differences between XY males and XX females by inactivating one copy of two X chromosomes (Nguyen & Disteche, 2006). XCI is generally proposed to occur in two stages: initiation and maintenance. The future inactive X-chromosome might undergo several different status of activity during initial phase. However, once established, the inactive state is stably maintained through multiple rounds of cell divisions. The establishment of inactive X chromosome can affect the progress and phenotypes of X-linked genetic diseases.

The oocyte is released from the ovary by ovulation in human. Fimbria, which is the terminal portion of fallopian tube, sweeps it into the oviduct. In the ampulla portion of the tube, fertilization occurs and embryo development begins with it. Early embryo development is a complex and sequential maturation process that consists of several stages, such as 1-cell, 2-cells, 4-cell, 8-cell, morula, blastocyst and epiblast. Although there are differences in early development between human and other mammals, there are still many similarities among them. Thus, mice have been the favored model to examine early embryogenesis including XCI due to their easy accessibility. Most fundamental findings of XCI described below were made in mice.

There are three different forms of XCI in embryogenesis of eutherian mammals. One is imprinted paternal X inactivation, another is a random X inactivation, and the other is a meiotic sex chromosome inactivation (MSCI). These different types of XCI are adopted in different stages of development (Fig. 1). Initially, X inactivation is imprinted with the exclusive inactivation of paternal X chromosome before implantation (Kay et al., 1994). At around the implantation period, the silent state of X-chromosome is maintained in extra-embryonic tissues such as trophoectoderm. However, the inactive paternal-X chromosome becomes reactivated in the inner cell mass (ICM) (Mak et al., 2004). Then, these cells undergo random X-inactivation of either the maternal- or the paternal-X individually, thereby resulting in females being mosaic for X-linked gene expression (Monk & Harper 1979; McMahon et al., 1983). Meiotic sex chromosome inactivation occurs in male spermatogenesis.

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Sex chromosomes are considered to have evolved from a pair of autosomes. One of those which has gained the sex-determining gene degraded and the dosage of genes on the sex chromosomes became different between the sexes (Marin et al., 2000). To compensate for the difference, various mechanisms have been used by different animals (Meyer, 2000; Akhtar, 2003). XCI has mainly been observed in mammals. Genomic imprinting by which the expression of genes is restricted to one parental allele is involved in inactivating X-chromosome. Epigenetic regulation is a useful mechanism for cells to inherit their identity to the descendents without changing the underlying DNA sequences. Both genomic imprinting and XCI are regulated by epigenetic modifications of genes. Therefore, investigating the mechanism of XCI provides the deeper understandings of chromosomal silencing, epigenetic regulation of gene expressions, and even the course of evolution.

This review covers the general mechanism of XCI taking place in early embryogenesis and describes the XCI according to the developmental stages. As a tool of studying XCI *in vitro*, we will describe the change in XCI during differentiation of embryonic stem cells (ESCs) and reprogramming of differentiated cells into induced pluripotent stem cells (iPSCs).

## OVERVIEW: MOLECULAR MECHANISM RELATED TO X-CHROMOSOME INACTIVATION

Triggering X-chromosome inactivation is regulated by a region on the X-chromosome referred as the X inactivation center (Xic) (Rastan, 1983). It has been proposed that more than two copies of Xic in trans are needed to initiate random XCI (Gartler & Riggs, 1983; Augui et al., 2007). The Xic carries Xist gene, which is located in the long arm of the X chromosome in human. Murine chromosomes are consisted of only one arm, and murine Xist is in the

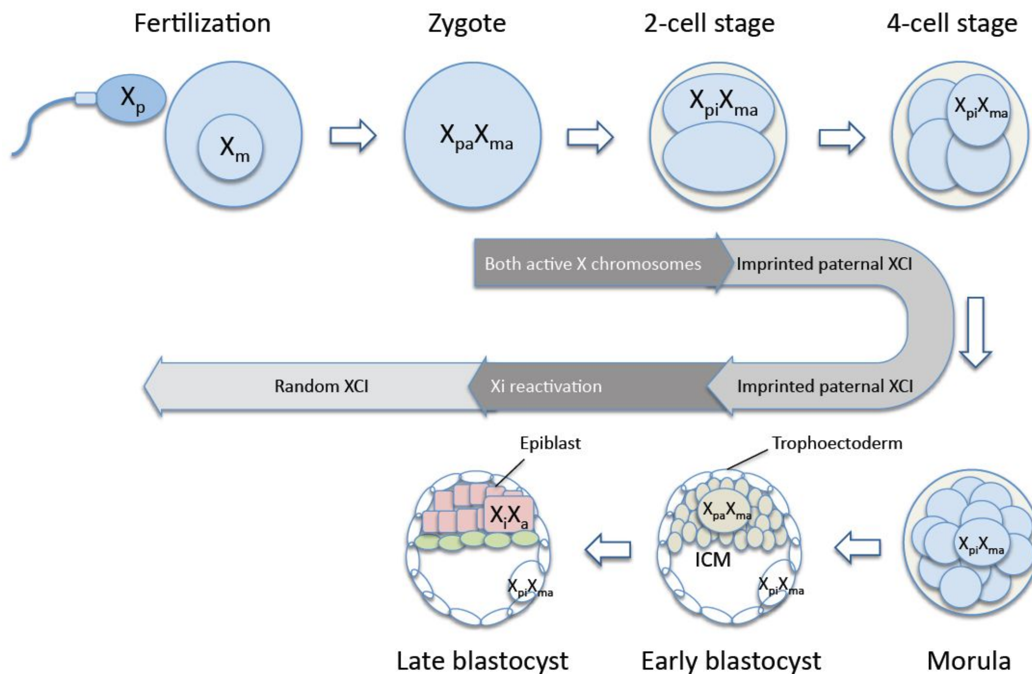


**Fig. 1. Dynamics of X chromosome inactivation (XCI) during mouse early embryogenesis.** X chromosome inactivation begins during 2-4 cell stages. XCI during this period occurs in paternal X chromosome ($X_p$) exclusively. Once established, $X_p$ inactivation maintains in extra embryonic tissues such as trophoectoderm. However, inactive $X_p$ in ICM of blastocyst reactivates. Then, random XCI takes place in ICM. The inactive state is stably maintained and transmitted through multiple rounds of cell divisions.

middle of X chromosome (Brockdorff et al., 1992; Brown et al., 1992). Xist produces a long non-coding RNA (ncRNA) that coats the chromosome from which it is transcribed (Plath et al., 2002). Xist is monoallelically upregulated before the initiation of XCI. Xist RNA expression and accumulation on the presumptive inactivative X chromosome (Xi) is followed by a series of histone modifications, methylations and chromatin changes on the future Xi (Monk & Harper, 1979; Chaumeil et al., 2004). After spreading along the presumptive Xi in cis, Xist RNA accumulates and recruits a protein complex responsible for chromatin changes on the future Xi (Zhao et al., 2008). Xist RNA is considered to attract several histone modifying enzymes, such as histone deacetylase, the polycomb repressive complex 2 (PRC2), the H3K27 and H3K9 histone methyltransferases. These enzymes mark the future Xi with repressive histone modifications, such as H3K27 and H3K9 methylation (Chaumeil et al., 2004). As a result, Xi becomes the heterochromatic chromatin structure, which does not permit the transcriptional machinery accessible for transcription on the Xi. Xist definitely affects silencing of X-chromosome in female eutherian mammals. However, recent studies showed that this effect is regarded to be dependent on local chromatin status (Chow et al., 2010; Tang et al., 2010).

There are various molecules mediating Xist regulation. While the Xi is distinguished by Xist expression, the active X chromosome (Xa) is characterized by the expression of the Xist anti-sense non-coding transcript (Tsix) which spans the entire Xist locus (Lee et al., 1999). Before the initiation of XCI, Tsix is expressed from both X chromosomes, thereby preventing Xa to be inactive. Although the exact mechanism of Tsix on Xist expression remains to be investigated, Tsix seems to repress Xist expression in cis by modulating Xist chromatin and determines which X chromosome would become inactive without affecting silencing (Navarro et al., 2005; Sado et al., 2005). Tsix expression links to the future active X chromosome and persists until Xist is silenced. Any alteration in Tsix expression leads to skewed XCI with preferential silencing of the X chromosome expressing lower levels of Tsix (Lee et al., 1999).

A cell needs to recognize the number of X chromosomes in it. Stable XCI occurs only if the number of X chromosomes exceeds one per diploid set of autosomes (Clerc & Avner, 2003). The X-pairing region (Xpr), a region on the X chromosome including Xist and Tsix is considered to mediate this counting through the homologous physical pairing between two Xics on each X chromosomes before Xist activation (Bacher et al., 2006; Augui et al., 2007; Xu et al., 2007; Monkhorst et al., 2008). However, the process in which the cell counts the number of X chromosome cannot be explained completely with the pairing of Xic. Recently, Ring finger protein 12 (Rnf12) gene in eutherian mammals has been proposed as the factor involved in counting mechanism (Jonkers et al., 2009). Rnf12, which is an E3 ubiquitin ligase, functions as a XCI-activator. Because Rnf12 is present on X chromosome, Rnf12 protein level significantly increased in female cells with two active X chromosomes. The increased Rnf12 induces Xist expression in a dose-dependent manner during random XCI above a certain threshold (Shin et al., 2010). The exact mechanism of activating Xist by Rnf12 remains to be investigated, but degradation of Xist repressor by Rnf12 was one of the plausible mechanisms (Fig. 2).

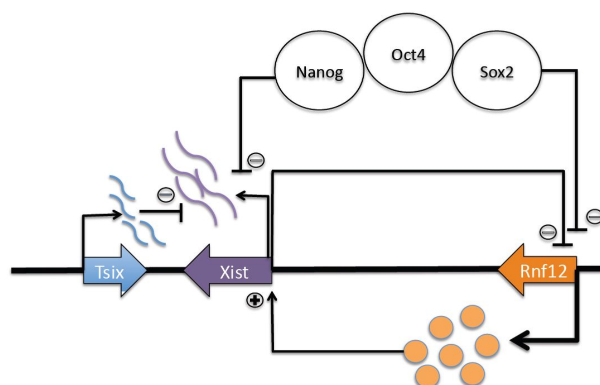


**Fig. 2. Xist regulatory network in X chromosome inactivation (XCI).** Xist is repressed by Tsix and several pluripotent factors, such as Nanog, Oct4, Sox2. Xist is activated by Rnf12 in dose dependent manner. Reprogramming factors suppress Rnf12 expression, reactivating Xi.

## IMPRINTED PATERNAL X-CHROMOSOME INACTIVATION

A zygote that results from the union of an oocyte and a sperm, is the earliest developmental stage of the embryo. After fertilization, the developmental process is driven by maternally inherited transcripts and proteins. The transition from the maternal to the zygotic transcription starts at late 1-cell stage or early 2-cell stages (Bouniol et al., 1995). This transition referred as maternal-zygotic transition. Then, zygotic gene activation (ZGA) takes place because the oocyte becomes transcriptionally silent (Bachvarova, 1985). At the time of ZGA, both X chromosomes are in active states (Fig. 1). However, paternal X chromosome (Xp) rapidly initiates XCI by the imprinted Xist expression from the 2-4 cell stages and the inactivation process is completed by the blastocyst stage. All cells undergo the imprinted paternal X chromosome inactivation during pre-implantation stages of early development. The inactivated Xp is maintained in the trophectoderm and primitive endoderm that will give rise to extraembryonic tissues, but is reactivated in the ICM (Chaumeil et al., 2004; Mak et al., 2004; Patrat et al., 2009).

For imprinted Xp inactivation, a maternal pool of Rnf12 is required in initiating the process (Shin et al., 2010). Retained pooling of Rnf12 is considered to activate Xist from imprinted Xp. Xist is exclusively expressed from the Xp (Ariel et al., 1995; Sado et al., 2001). From the active maternal X chromosome (Xm) during the imprinted Xp inactivation, Xist is suppressed by the repressive imprint deposited egg maturation (Tada et al., 2000). On the other hand, Tsix is transcribed on the Xm, which prevents inactivation of the Xm (Kay et al., 1994; Goto & Takagi, 2000). The inactivated Xp is reversed by reactivation in ICM. The Xist expression is down regulated and the proteins associated with heterochromatin, histone modifications and chromatin changes disappear (Chaumeil et al., 2004; Mak et al., 2004). Shortly after the reactivation period, random X inactivation take places in the epiblasts of early postimplantation embryo.

## RANDOM X INACTIVATION

As mentioned above, random X inactivation is controlled by the Xic. Several factors, such as the homologous pairing of Xpr between active Xp and Xm and the increased Rnf12 induce monoallelic X chromosome inactivation. In Xi, Xist, which is a long-ncRNA, coats and accumulates on the future Xi in cis. Accumulating Xist recruits various proteins that induce conformational change of chromatin, which blocks the transcription from Xi. On the other hand, in future Xa, Tsix, the antisense ncRNA of Xist, expresses and degrades the Xist. Therefore, Xa remains to be in an active state. The state of X chromosome is stably maintained through several rounds of cell divisions.

Imprinted and random XCI share many features in the mechanism of X chromosome inactivation. Xist and polycomb group proteins are involved in inactivating X chromosomes. In addition, Tsix transcription is the hallmark of the retaining activity of X chromosome in both imprinted and random XCI. However, there are fundamental differences between two the two XCI. Imprinted XCI do not require the process of counting the number of X chromosomes, because Xist is expressed from paternal X chromosome only. In random XCI, the imprint of X chromosomes is lost and the XCI needs several mechanisms, such as X chromosome homologous pairing and Rnf12 expression to recognize the number of X chromosome.

## X CHROMOSOME REACTIVATION (XCR) DURING REPROGRAMMING

The study about XCI using mouse embryonic stem cells (mESCs) confirmed once again that the differentiation is associated with random XCI in mice (Chaumeil et al., 2004). Nichols and Smith proposed two phases of pluripotency in stem cells referred as naive and primed (Guo et al., 2009). The naïve state represents a fully unrestricted state that harbors the flexible developmental potency to produce all embryonic lineages. The cells of ICM in blastocyst are in

naïve state and mouse mESCs derived from ICM represent the naïve state. mESCs share epigenetic features and X chromosome states with preimplantation naïve ICM. Naïve ICM cells become primed for lineage specification upon external stimuli. Cells in late epiblasts are in primed state and so is their *in vitro* counterpart, mouse epiblast stem cells (mEpiSCs) (Brons et al., 2007). mEpiSCs only show one active X chromosome in females, while mESCs have two active X-chromosomes. In mice, these two phases are interconvertable (Chou et al., 2008). In addition, the X chromosome silencing can be reverted through reprogramming process from mEpiSCs into EpiSC derived induced pluripotent stem cells (Guo et al., 2009). Murine iPSCs also harbors two active Xs and subsequently random XCI occurs during *in vitro* differentiation (Maherali et al., 2007).

In undifferentiated mESCs, the strong transcriptional repression of Xist by pluripotent genes ensures the active states of two X chromosomes. Likewise, reprogramming of differentiated cells into iPSCs reactivates the inactive X chromosome (Chou et al., 2008). The depletion of Nanog, Oct4, or Sox2 in mESCs triggers rapid ectopic accumulation of Xist RNA, while during reprogramming Oct4, Nanog and Sox2 are involved in Xist repression. The direct bindings at the first intron of Xist by Nanog, Oct4 and Sox2 was noted as a plausible mechanism of repression (Navarro et al., 2008). Hower, the deletion of the intron 1 region of Xist did not affect the Xist repression in undifferentiated mESCs, suggesting that mechanisms other than the binding at first intron of Xist by pluripotent genes are involved in Xist repression (Barakat et al., 2011). Despite the exact mechanism remains to be determined, these results showed that X chromosome status is closely regulated by pluripotency machinery in mice.

Tsix is involved in the maintenance of low levels of Xist in undifferentiated mESCs. The pluripotent marker reduced expression 1 (Rex1), and reprogramming factors c-Myc and Klf4 showed the activation and elongation of Tsix by binding to the DXPas34 minisatellite associated with Tsix promoter (Navarro et al., 2010). The binding of these factors in the Tsix promoter seems to induce the remodeling the chromatin in the locus. Although the relationship between the pluripotency and X chromosome status is well established during embryonic development and ESC differentiation or iPSC formation, it is still unclear whether the presence of active X chromosomes is a direct or indirect effect of the establishment of the pluripotent state.

## DIFFERENCES OF XCI BETWEEN MICE AND HUMAN X CHROMOSOME

Investigating XCI in human developments has many barriers, such as ethical issues and technical difficulties dealing with human preimplantation embryos. Thus, investigation of XCI in mammals was performed in mice as a model, but recent studies showed that there are major difference of XCI between mice and human, especially in imprinted paternal XCI. Indeed, the existence of imprinted XCI in human still remains controversial. Recent study showed that XCI in human placental tissues is random with either maternal or paternal X chromosome being inactive (Moreira de Mello et al., 2010). Another study revealed that Xist homologue in human is not imprinted and chromosome wide XCI do not initiated by the blastocyst stage although XIST is upregulated (Okamoto et al., 2011).

In addition, TSIX is considered to be transcribed from only Xi together with XIST, and does not repress the XIST (Migeon et al., 2001; Migeon et al., 2002). These demonstrate the remarkable diversity in the mechanism of XCI between mammals. To investigate the XCI in human, human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) would be a readily available and more proper *in vitro* experimental model.

## X CHROMOSOME INACTIVATION AND REACTIVATION IN HUMAN

In hESCs, X chromosome status varies according to the cell lines and culture conditions (Hoffman et al., 2005; Adewumi

et al., 2007; Shen et al., 2008). Current culture conditions does not seem to maintain hESCs with stable X chromosome status. Many features of hESCs, such as morphology and signaling pathway for self-renewal suggest that hESCs represent murine counterpart of epiESCs that are developmentally more advanced than mESCs. Thus, most hESCs were shown to be at the primed state. Recently, hESCs in a naïve state that express two Xa were successfully derived using the modified culture condition under physiologic oxygen concentration (Lengner et al., 2010). The authors further demonstrated that differentiation of hESCs results in random XCI similarly as in mESCs. These results demonstrate that the human blastocyst contains pre-X-inactivation cells, and Xi state is stably maintained *in vitro* under proper culture condition (Lengner et al., 2010). However, stresses such as long-term culture, oxidative stress and freezing/thawing, cause XCI in biallelic hESCs, suggesting that X chromosome in hESCs are generally in unstable status.

Reprogramming human somatic cells into iPSCs also provides an excellent alternative for elucidating XCI mechanisms in human cells. Compared with that of mouse, the status change in X chromosome during human somatic cell reprogramming is less clear. Tchieu et al, generated hiPSCs from several female fibroblasts under standard culture conditions (Tchieu et al., 2010). Although the X chromosomes in established hiPSCs showed the chromatin changes, XCR did not occur. Recent study also showed that XCR does not take place in reprogramming of female fibroblasts from Rett syndrome patients (Pomp et al., 2011). However, Marchetto et al reported conflicting results by generating hiPSCs with two active X chromosomes from female Rett patients (Marchetto et al., 2010). Complementing both reports, our group generated hiPSCs that retained the inactive X chromosome of fibroblast as well as those that reactivated the inactive X chromosomes (Kim et al., 2011). There are several possible reasons for discrepancy among the results, such as reprogramming method, medium conditions, or feeders. Detailed comparison among reprogramming methods to generate hiPSCs and X chromosome status will be needed to resolve the difference.

## FUTURE PERSPECTIVE

Indeed, our understanding of XCI has increased remarkably over the past decades mainly during early mouse development and ESC differentiation. Recent analysis of the X chromosome status during somatic cell reprogramming enables the investigation of the novel aspect of XCR. However, the molecular mechanism of somatic cell reprogramming and interconnection between pluripotency and XCI/XCR still remain to be explored. First of all, in-depth study of XCI in human cells should be performed. So far, most basic molecular mechanism of XCI have come from murine model systems. Murine XCI data will definitely help elucidating the human XCI, but more emphasis on studying human XCI is needed. Secondly, the difference among different types of XCI, which are random XCI, imprinted XCI, and meiotic sex chromosome inactivation, is investigated. Thirdly, the molecular pathway leading to X chromosome reactivation is one of the interesting subjects. Transition from morula to blastocyst stage, and primordial germ cell development accompany the *in vivo* X chromosome reactivation. Especially, reprogramming of female somatic cells may provide the important information understanding the fundamentals of XCR. Fourthly, a subset of X-linked genes escapes silencing of XCI and expressed from both X chromosomes. Studies about the mechanism allowing the XCI escape are needed to further characterize the chromatin structure of escape domains.

Investigating XCI is one of the most fascinating topics of research. Unraveling the mechanisms underlying the developmental regulation of XCI, and particularly of Xist, has been a long-standing, and still ongoing, challenge. Studying XCI will allow us to better understand the fundamental biology of chromatin regulation, DNA methylation, early embryogenesis and evolution process, but also to develop therapies for X linked genetic diseases, such as Rett syndrome, Duchene muscular dystrophy, and alpha-

thalassemia (Kim et al., 2011).

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